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Morphine and other analgesics bind to and activate the μ-opioid receptor (MOR). The opioid receptors belong to the Class A subfamily of G-Protein Coupled Receptors (GPCRs). These are transmembrane proteins with seven helices arranged to form a closed bundle with loops that extend both extracellularly and intracellularly. Activation of this family of GPCRs has been shown to involve the change of the χ1 dihedral of a tryptophan residue on TMH6, W6.48, from g^+ to *trans*. The purpose of this project was to design a computational model of the MOR and to dock both morphine an agonist, and naloxone an antagonist, into the model such that their positions were consistent with their pharmacologies.

A MOR model was created using the Beta-2-Adrenergic (β 2-AR) crystal structure as a template with two major modifications. First, the Conformational Memories (CM) program was used to study the conformations of three transmembrane helices (TMH); TMH2, TMH4 and TMH6. Second, the TMH7/elbow/Hx8 region of the β 2-AR was replaced with that of the adenosine A2A crystal structure because the adenosine A2A receptor has the same number of residues in the elbow region as is found in the MOR. Energy minimizations were performed on the MOR bundle in a three step process and the ligand binding pocket was identified. Docking studies suggested that naloxone binds in the TMH2-3-6-7 region of the MOR such that the N-allyl group sterically prohibits the movement of the χ1 of W6.48, thereby preventing activation of the

receptor. Morphine was also found to bind in the TMH2-3-6-7 region of the MOR; however no portion of the morphine structure could block the movement of the $\chi 1$ of W6.48, thereby producing no impediment for activation. These results are consistent with the pharmacology of naloxone (MOR antagonist) and morphine (MOR agonist). Models created will be used for future mutation studies.

CONSTRUCTION OF A μ-OPIOID RECEPTOR MODEL:

IDENTIFICATION OF THE OPIOID ALKALOID

BINDING POCKET

by

Elizabeth B. Poole

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

For people suffering from many illnesses, pain management is often one of the most difficult aspects of treatment. Morphine and its derivatives are generally the drugs of choice of many physicians and for patients dealing with chronic pain. However, these medications have many side effects including respiratory depression, gastrointestinal problems as well as dependence and addiction. Consequently, the need for a pain relieving agent without these side effects is of utmost importance.

Morphine and other analgesics bind to and activate the μ-opioid receptor (MOR) (Raehal and Bohn, 2005). The opioid receptors, delta, kappa and mu, belong to the Class A subfamily of G-Protein Coupled Receptors (GPCRs). This subfamily is the Rhodopsin-like family and the dim light photoreceptor Rhodopsin is the base sequence typically used for homology modeling of other receptors in this class. GPCRs are transmembrane proteins with four distinguishing structural characteristics as illustrated in Figure 1:

1. Seven transmembrane α-helices (TMHs) arranged to form a closed bundle
2. An extracellular N-terminus
3. Extracellular (EC) and Intracellular (IC) loops that connect each of the seven TMHs

4. An intracellular C-terminus that begins with a short helix oriented parallel to the membrane (called Helix 8)

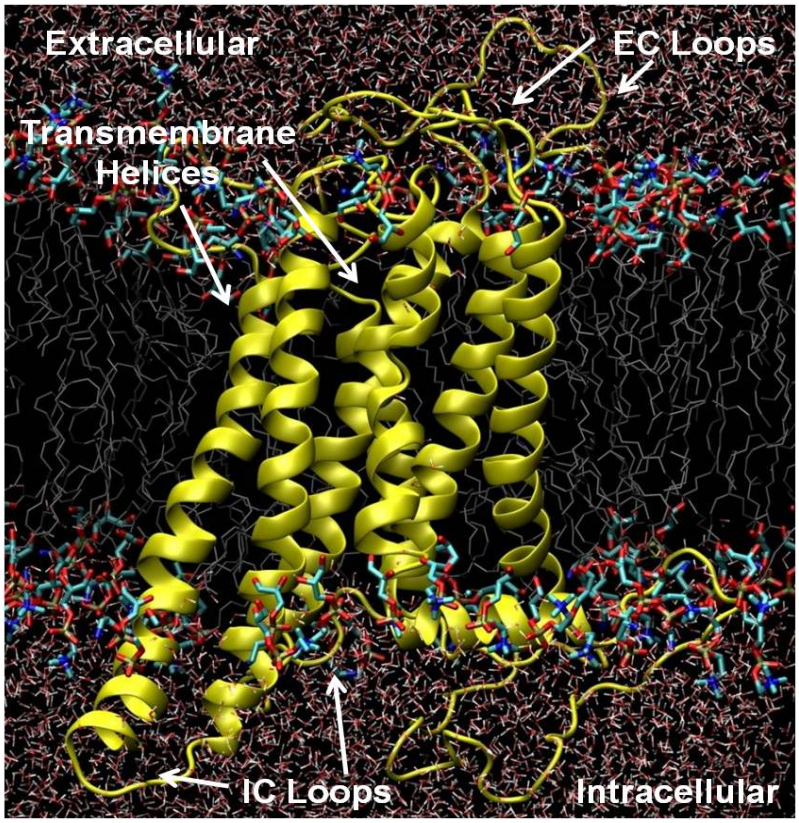


Figure 1 The structure of a prototypical Class A GPCR

Until recently, the only Class A GPCR x-ray crystal structure available was that of rhodopsin (Palczewski et al., 2000). In 2007-2008, x-ray structures became available for the carazolol bound beta-2-adrenergic (β 2-AR) (Cherezov et al., 2007; Rasmussen et al., 2007), as well as for the cyanopindolol bound β1-AR (Warne et al., 2008) and the ZM241385 bound adenosine A2A receptor (Jaakola et al., 2008). These structures

showed substantial similarity to the earlier rhodopsin structure, but important differences are present. One of the most striking differences can be seen in the positions of TMH1 and TMH2. The transmembrane helix bundle in the rhodopsin crystal structure is tightly packed with TMH1 and TMH2 pulled inwards towards the center of the bundle. TMH2 of rhodopsin has a GGXTXT motif which can accommodate larger phi/psi values. As a result, the threonines work with the glycines to support hydrogen bonding and bulky residues to help stabilize the helix and bundle (Palczewski et al., 2000). In the β 2-AR crystal structure (Krystek et al., 2006) TMH1 and TMH2 are pulled away from the bundle. This allows more room for both the binding pocket and for TMH7, which is pulled away in the β 2-AR bundle. The GGXTXT motif seen in rhodopsin is absent in β 2-AR, which instead has a proline at 2.59. Also, in rhodopsin, TMH4 has two prolines side by side at the extracellular end of the helix. However, TMH4 in the β 2-AR, like the mu-opioid receptor (MOR) has only one proline.

GPCR activation studies have primarily come from biophysical studies of rhodopsin and the β 2-AR receptor. In the inactive or off state (R), there is a salt bridge between R3.50 and E6.30 on the intracellular side of the TMH bundle that is called the "ionic lock". Previous studies indicate that this ionic lock is broken during activation as a direct result of TMH 3 and TMH 6 rotating away from each other (Ballesteros et al., 2001). In TMH 6 there is also a conformational change due to the helix straightening in the highly conserved CWXP hinge region (Farrens et al., 1996; Ghanouni et al., 2001; Javitch et al., 1997; Jensen et al., 2001; Lin and Sakmar, 1996; Nakanishi et al., 2006). Studies have shown that while the (MOR) does not form an ionic lock at the exact same

location as rhodopsin, it does form an ionic lock between R3.50 and T6.34 (Huang et al., 2002). In the inactive or R state, it has also been shown that the conformation of a tryptophan residue on helix 6, W6.48, is such that the χ^1 dihedral angle is in a g^+ conformation. This conformation in rhodopsin is due to the close proximity of the beta-ionone ring of rhodopsin's covalently bound ligand, 11-cis-retinal to W6.48, such that the tryptophan is locked in place (Li et al., 2004; Okada et al., 2002; Palczewski et al., 2000). Once light activates the receptor by isomerizing 11-cis retinal to all-trans-retinal, the beta-ionone ring moves away from TMH-6 and towards TMH-4, allowing the χ^1 of W6.48 to undergo a $g^+ \rightarrow$ *trans* conformational change (Shi et al., 2002). In the β 2-AR, Shi and co-workers showed that a rotamer "toggle switch" in TMH6 may modulate the proline kink differences in the R and R* bundles. This toggle switch is comprised of three residues on TMH6; C6.47, W6.48 and F6.52 in the β 2-AR. In the MOR, these residues correspond to C6.47, W6.48 and H6.52. The β 2-AR R state is characterized by these three residues having g^+*/trans/trans* χ^1 geometry, respectively. The β 2-AR R* state is characterized by these three residues having *trans*/g^+/g^+ χ^1 geometry (Shi et al., 2002).

The opioid receptors bind both peptide and non-peptide ligands. Naloxone is a classic (non-peptide) antagonist of the MOR (see Figure 2). As such, naloxone is currently in use worldwide for people who have overdosed on heroin, cocaine or pain killers such as morphine, oxycodone or hydrocodone. In the MOR, the classical μ agonist, morphine, is displaced by naloxone and the receptor is changed to an inactive or off state (R). Naltrexone is another MOR antagonist. The binding pocket for MOR non-

peptide ligands is commonly thought to be formed by TMH3, TMH5, TMH6, TMH7 and EC Loop 2 (Kane et al., 2006). However, site-directed mutagenesis studies have shown that a tyrosine residue (Y7.43) plays an important role in the binding affinity of both morphine and naloxone (Mansour et al., 1997). When residue 7.43 was mutated to a phenylalanine, both morphine and naloxone had decreased binding affinities, likely due to the loss of a hydrogen bond between Y7.43 and the ligands. Based on available models, using a rhodopsin template, this residue is not thought to be near the binding pocket predicted by Kane in 2006 (Hurst, 2008). As illustrated in Figure 2, naloxone and morphine are structurally very similar molecules with the largest difference being the N-17-substituent which is an N-methyl for morphine, but enlarged to an N-allyl in naloxone. The hypothesis to be tested in the work proposed here is that the origin of naloxone's MOR antagonism lies in its N-allyl group. This group may sterically block the W6.48 g^+ → *trans* conformational change associated with activation, thus restraining the receptor to its inactive R state.

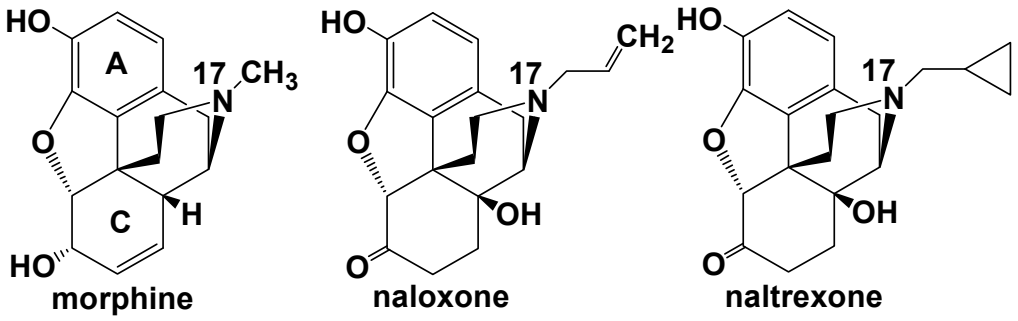


Figure 2 Structures of Morphine, Naloxone & Naltrexone

In the MOR, it has previously been shown that D3.32 serves as the counter-ion for charged amine containing ligands (Surratt et al., 1994). When the negative charge of the aspartic acid was lost upon mutation to an alanine or asparagine, both naloxone and morphine had decreased affinities for the receptor (Surratt et al., 1994). In the docking studies reported here, the negatively charged D3.32 will be used as the primary interaction site for morphine and naloxone.

A few years ago, the Ping-Yee Law lab at the University of Minnesota discovered single and triple mutations of the MOR at which naloxone behaves as an agonist with concomitant production of antinociception (i.e. pain relief) (Portoghese et al., 2003; Yang et al., 2003). The first mutant receptor discovered by the Law lab to have this property was a single mutant in which serine 196 (S4.54) was mutated to alanine (S4.54(196A)), resulting in partial agonism of naloxone (Yang et al., 2003) The second mutant receptor, was a triple mutant (Claude-Geppert et al., 2005); S4.54(196)A, T7.44(327)A, and C7.47(330)S, which resulted in full agonism of naloxone. Figure 3 illustrates the position of each of the mutated residues.

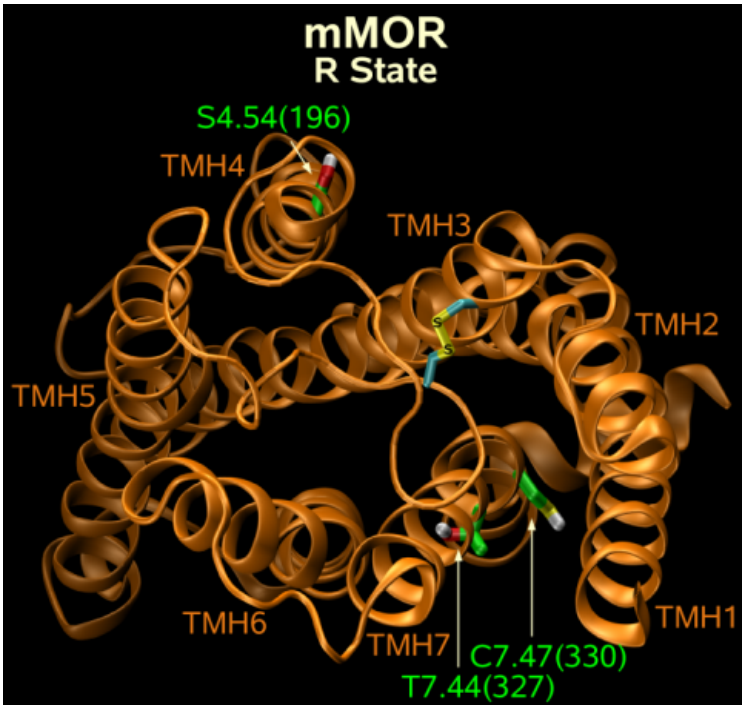


Figure 3: Locations of mutations in a triple mutant MOR

(Claude-Geppert et al., 2005)

Targeted gene therapy studies using the single mutant have indicated that naloxone can act as an antinociceptive agent at this mutant *in vivo* (Chen et al., 2007). Because expression of the mutant MOR is targeted to a limited region of the spinal cord, administration of naloxone results in native Wild Type MOR's being antagonized with only the localized mutant MORs being activated by naloxone. The reduced number of receptors activated in this paradigm results in no measurable dependence/addiction as seen with traditional mu agonists like morphine (Chen et al., 2007).

It is important to note that the S4.54(196)A and S4.54(196)A/ T7.44(327)A/ C7.47(330)S mutants each involve a serine or threonine and surprisingly that none of the mutated residues face into the MOR binding pocket (see Figure 3). Previously it has

been shown that serines and threonines can induce conformational changes in transmembrane helices. The hydrogen bonding capacity of Ser/Thr residues in α-helices can be satisfied by an intrahelical hydrogen bond interaction, in either the *g*- or g^+ conformation, between the O-γ atom and the *i-3* or *i-4* carbonyl oxygen (Ballesteros et al., 2000). Ser/Thr residues in the *g*- conformation can induce a bend in an α-helix, and we have found that changes in wobble angle and face shift can also occur (Ballesteros et al., 2000). The work described here is intended to serve as groundwork for the ultimate determination of the molecular origins of the unusual phenotype of this triple MOR mutant.

CHAPTER II

HYPOTHESIS & METHODS

Hypothesis to Be Tested

This project focused on designing a computational model for the wild-type (WT) MOR using current GPCR crystal structures and their structural motifs as templates. The β 2-AR and the adenosine A2A structure played a large role in the model as they were the template for transmembrane helices 1-6 (β 2-AR) and helix 7 / elbow region / helix 8 (adenosine A2A). The binding pocket was identified and both morphine a mu opioid agonist, and naloxone, an antagonist were docked in the resultant model. Interactions that play a role in the binding of both ligands were analyzed to determine key binding pocket residues. The main hypothesis to be tested in the work presented here is that the N-allyl group of naloxone sterically blocks the W6.48 $g^+ \rightarrow$ *trans* conformational change associated with activation, thus restraining the receptor to its inactive R state. This model will be used in future projects including a project in conjunction with Dr. PY Law to determine the molecular origins of the unusual phenotype of the S4.54(196)A/ T7.44(327)A/ C7.47(330)S MOR mutant in which naloxone, the classical MOR antagonist becomes an agonist (Claude-Geppert et al., 2005).

Methods

Initial Model Construction

The rhodopsin x-ray crystal structure (Palczewski et al., 2000) was originally used as the template for building an MOR model. However, for reasons discussed below, it was concluded that the β 2-AR x-ray crystal structure is a better template (Krystek et al., 2006). The initial MOR model was constructed by first aligning the μ sequence with that of the template using the highly conserved Class A GPCR sequence motifs (TMH3 E/DRY; TMH6 CWXP; TMH7 NPXXY) or highly conserved residues (N1.50, D2.50, W4.50, P5.50) as the alignment guides. The structure of the template was mutated to the corresponding mu residue using Maestro (Schrodinger 2006). MOR helices were then screened for the occurrence of helix deforming residues such as prolines or glycines that may cause the MOR helix conformation to deviate from that of the template. The conformations possible for such helices were studied using Conformational Memories (see below) and an appropriate substitute helix was incorporated into the model.

After developing a model based on the β 2-AR crystal structure, it was determined that the TMH7, elbow region and Hx8 area needed to be adjusted. The β 2-AR structure only has one residue in the elbow region, whereas the MOR has 2 residues. To match this structural motif in the MOR, the TMH7/elbow/Hx8 region of the adenosine A2A receptor was used as the model template (Jaakola et al., 2008). Therefore the final MOR model was constructed using a β 2-AR template for helices 1 through 6 and the adenosine A2A template for helix 7, the elbow region and helix 8.

Amino Acid Numbering System

In work reported here, the amino acid numbering system proposed by Ballesteros and Weinstein (Ballesteros et al., 2001) is used. In this system, the most highly conserved residue in each transmembrane helix is assigned a locant of 0.50. This number is preceded by the helix number and can be followed by the sequence number in parentheses. All other residues are numbered relative to this residue. For example, the most highly conserved residue in TMH2 is D2.50.The alanine residue that immediately precedes it in the MOR would be numbered A2.49, while the alanine immediately after it would be numbered A2.51.

Torsion Angle Definition

The nomenclature used herein to describe the rotameric state of the χ1 side chain torsion angles is what was described by Shi and co-workers (Shi et al., 2002). A residue is described to have a *trans* χ1 angle when the location of the heavy atom at the γ position is opposite the backbone nitrogen (180°). A residue is described to be *gauche*$^{+}$ *(g*$^{+}$*)* when the γ heavy atom is opposite the backbone carbon and has an angle of +60° when viewed from β-carbon to the α-carbon, while it is described as a *gauche*$^{-}$*(g*$^{-}$*)* χ1 angle when the γ heavy atom is opposite the α-hydrogen when viewed from the β-carbon to the α-carbon at -60° (Kapur et al., 2007).

Conformational Memories (CM)

The conformational memories (CM) method was used to explore the possible conformations of MOR helices that contain helix deforming residues as positions not found in the template structures (Konvicka et al., 1998). This method employs multiple Monte Carlo/simulated annealing random walks and the CHARMM force field (Konvicka et al., 1998; Whitnell et al., 2008). The CM method has been shown to converge in a practical number of steps and to be capable of overcoming energy barriers efficiently. With the CM method, the conformational properties of helices can be fully characterized based on their free energies. This includes both the intrinsic energy of each conformational state and the probability that the helix will adopt a specific conformation relative to the other accessible conformations in an equilibrated thermodynamic ensemble. This technique explores the low free energy conformations possible for a helix of interest using Monte Carlo simulated annealing of side chain dihedrals and bond angles, while the backbone dihedrals of each helix were set to the standard φ (-63°) and ψ (-41.6°) for transmembrane helices (Ballesteros et al., 2000). In the Reggio lab, the established protocol is to allow all torsion angles to vary ±10°, and to allow a larger variation of ±50° in regions containing flexible areas. These flexible areas are areas where there are known helix bending residues such as prolines, glycine's, serines and/or threonines (Visiers et al., 2000). Individual bond angles are allowed to vary ±8. The CM calculation is performed in two phases as described below.

Exploratory Phase

In the CM method, a random walk is used to first identify the region of conformational space most probable for each torsion angle and bond angle. The initial temperature for each run is 3000 K with 50,000 Monte Carlo steps applied to each torsion or bond angle variation with cooling in 18 steps to a final temperature of 310 K. Each step consists of varying two dihedrals angles and one bond angle chosen at random from the entire set of variable angles. The torsion angles and bond angles are randomly picked at each temperature and each move is accepted or rejected using the Metropolis criterion (Konvicka et al., 1998). Accepted conformations in the Exploratory Phase are used to create "memories" of torsion angles and bond angles that were accepted. This information provides a map of the accessible conformational space of each TMH as a function of temperature.

Biased Annealing Phase

In the second phase of the CM calculations, the only torsion angles and bond angles moves attempted are those that would keep the angle in the "populated conformational space" mapped in the exploratory phase. The biased annealing phase begins at 749.4 K with cooling to 310 K in 7 steps. Finally 105 structures are output at 310 K.

Superimposition and Determination of Helices

In each study, all 105 output conformers for an individual TMH were superimposed onto the corresponding template helix in the model. For all superimpositions described herein the C, Cα and backbone N were used as the basis for the superimposition. The specific ranges of residues superimposed are described in the next section. An appropriate helix conformation was selected based upon the absence of Van der Waals overlaps with other helices in the bundle, and upon the appropriate wobble angle to accommodate loop inclusion. The helices studied in Conformational Memories were superimposed as described in the next section.

Helices Studied Via Conformational Memories

Models of TMH2, TMH4 and TMH6 were created using the conformational memories (CM) technique.

TMH2: All GPCR's are aligned using D2.50 in helix 2 as the alignment guide for TMH2. There is also a proline residue at position 2.58 or 2.59 in most Class A GPCRs. In the μ sequence, the proline is located at position 2.58, but in the β 2-AR template sequence it is at position 2.59. This offset by one residue is enough to result in a different conformation of TMH2 relative to the β 2-AR template. To ensure a correct conformation of TMH2, CM was used. The region of i (P2.58) to i-4 (T2.54) was considered to be the flexible area. The residues in this area were allowed to vary by

±50°. The output structures were superimposed onto the β 2-AR template using the C, Cα and backbone N atoms of residues A2.38 through L2.57.

TMH4: In the MOR sequence there is a proline at residue 4.59, however, in the β 2-AR sequence the proline is located at 4.60. As a result, the Conformational Memories technique was used to determine a wild-type TMH4 for the current work. The area allowed to vary ±50° was I4.51 to P4.59. Output CM structures (work done by Hadley Iliff) were superimposed onto the β 2-AR template using the C, Cα and backbone N atoms of residues R4.40 through N4.49 and a wild-type TMH4 was selected and incorporated into the current model.

TMH6: TMH6 of the MOR contains the Class A GPCR CWXP flexible hinge motif. Previous studies have suggested that the W6.48 χ^1 is in a g^+ position in the R state, and in *trans* in the R* state (Ghanouni et al., 2001; Javitch et al., 1997; Jensen et al., 2001). An ionic lock has been shown to exist between TMH3 and TMH6 in the R state. This lock has been shown to be constituted by R3.50 and T6.34 in the MOR R state (Huang et al., 2002). Conformational Memories was used to select an appropriate helix for the wild-type model (R state). The *i* (P6.50) to i-4 (V6.46) region was allowed to vary by ±50°. Helices were separated based on the χ1 conformation of W6.48 and the output structures with a χ1 in the g^+ conformation were superimposed onto the β 2-AR template from R6.31 through V6.46 using the C, Cα and backbone N atoms.

TMH7: In Class A GPCRs, TMH7 has a conserved sequence motif of NPXXY. The conserved P is located at 7.50. The flexible region allowed to vary ±50° in TMH7 was Y7.43 to S7.47. The CM output structures were superimposed onto the β 2-AR template from T7.44 to L7.56 using the C, Cα and backbone N atoms. However, as discussed in Chapter III, ultimately the output from CM for TMH 7 had to be discarded and the adenosine A2A crystal structure was used as the template for TMH 7.

Building the Bundle & Minimizations

Building the Bundle

To build the wild-type MOR, the β 2-AR crystal structure bundle was mutated to the MOR sequence. The CM output helices that had been selected were superimposed accordingly, and the bundle was minimized in three steps as described below. Once it was determined that the TMH 7/elbow/Hx 8 region needed to be adjusted, the corresponding area of the adenosine A2A bundle was superimposed on the β 2-AR template, and then mutated to the MOR sequence. The bundle was minimized as before.

Minimizations

Before minimizing, each bundle was pulled apart 3Å away from TMH 3 to allow room for the side chains to accommodate each other in the new bundle. Each residue was adjusted manually to its most energetically favorable position, while allowing room for all other residues and preventing any Van der Waals conflicts. The χ1 torsion angles of C6.47, W6.48 and H6.52 were adjusted to *trans*/g^+/g^+ respectively to be in agreement

with the “toggle switch” proposed by Shi and co-workers (Shi et al., 2002). Also, R3.50 and T6.34 were oriented towards each other to promote the formation of the ionic lock when the bundle was minimized (Huang et al., 2002). Once all Van der Waals overlaps had been relieved, the minimization was started using an OPLS_2005 force field, no solvent and a distance dependent dielectric. The minimization was set to converge to 0.05kJ/mol·Å^2. Once a converged output structure was achieved in step 1, the output was used as the input for step 2. This was repeated for steps 2 and 3. The final structure used was the converged structure from the third step of the minimization.

Step 1: All backbone phi / psi dihedrals were constrained with a force of 500kJ/mol. The distance between the first nitrogen of the guanidine group of R3.50 and the hydroxyl oxygen of T 6.34 was constrained to a distance of 2.8Å ± 0.4Å with a force of 15kJ/mol. The amino acid side chains of each residue were allowed to vary.

Step 2: All backbone phi / psi dihedrals were constrained with a force of 100kJ/mol. There were no constraints placed on any distances or other angles, and all side chain dihedrals were allowed to vary.

Step 3: The backbone phi / psi dihedrals had no constraint placed on them. All backbone dihedrals as well as all side chain angles were allowed to vary.

Docking Methodology

Before docking morphine or naloxone, an AM-1-conformational search was be performed to identify the global minimum energy conformation of each. The global min was then used for docking studies. In the MOR, Asp3.32 was used as the primary interaction site. Each ligand was docked using interactive computer graphics via Maestro (Schrodinger, 2006). The resulting ligand/receptor complex was energy minimized using CHARMM, in the same 3 step process as described above under minimizations for the unoccupied receptor.

Interaction Energy Calculation

The interaction energy between the ligand (morphine or naloxone) and the minimized receptor complexes were calculated using a Molecular Mechanics tool in Maestro (Schrodinger, 2006). The OPLS_2005 force field was used, with no solvent and a distance dependent dielectric. The ligand was identified manually (selected by hand), and all residue interactions within 5Å of the ligand were used in the calculation.

CHAPTER III

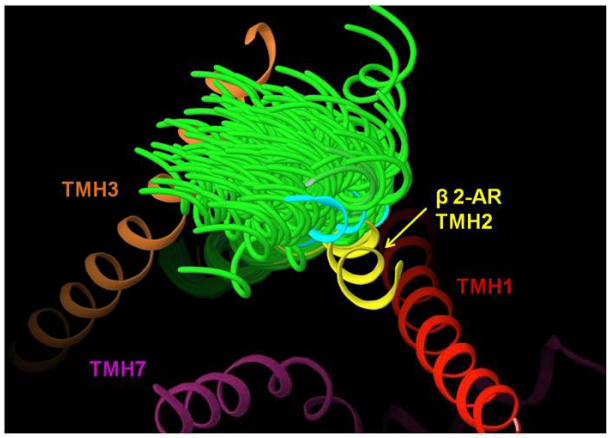
RESULTS

Conformational Memories (CM) Output

TMH2

The β 2-AR receptor and the MOR both lack the GGXTXT motif found in Rhodopsin. Each has a proline near its extracellular end; however this proline is at 2.59 in β 2-AR and 2.58 in the MOR. To allow for this difference, CM was used to study the conformations of TMH2 in the MOR, and the output structures were superimposed onto the β 2-AR template.

Figures 4a and 4b show the superimposition of the CM output structures for TMH2 onto the β 2-AR template. The yellow helix is the β 2-AR TMH2, while the lime green helices are the CM output structures. It can be seen in Figure 4 that the location of the proline in the μ-opioid helices does cause the top (extracellular) end of TMH2 to kink back to the interior of the bundle, however, the direction of the kink causes the extracellular end of TMH2 to move towards TMH3 in the MOR instead of TMH1 as seen in the β 2-AR crystal structure. This helix (cyan) does not have any Van der Waals overlaps that would prevent it from fitting into the MOR bundle, and the selected helix is slightly pulled out at the extracellular end.



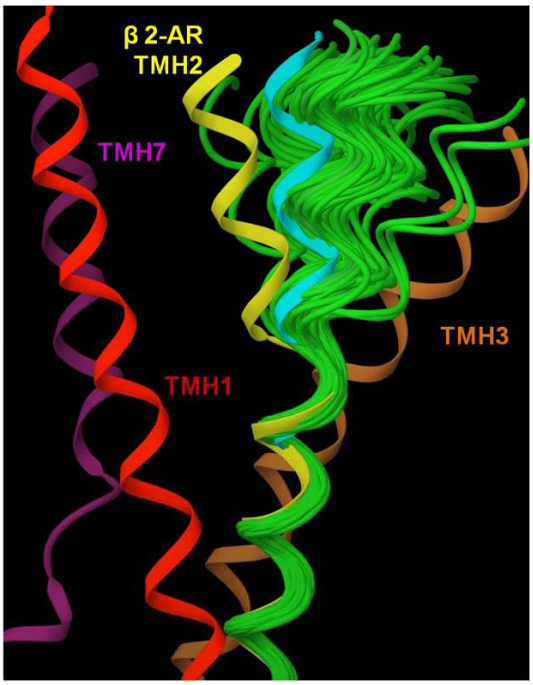


Figure 4: CM output for TMH2 superimposed on the β 2-AR template. 4a (left) shows the CM output for TMH2 superimposed on the β 2-AR template from an extracellular view. 4b (right) shows a side view from lipid.

TMH 4

A wild type TMH4 was selected from the CM output structures initially calculated by Hadley Iliff. While this helix did fit in the initial Rhodopsin template of the wild-type MOR, it did not fit in the β 2-AR template due to clashes at the extracellular end of the bundle between TMH3 and TMH4. The second TMH4 chosen was initially thought to be a possibility due to the extracellular end being pulled away from TMH3, however there were steric interactions that prevented it from working as well. Also, V4.60 sterically forced the χ1 angle of Y3.33 into a g^+ conformation, thus not allowing it any freedom to move into a *trans* χ1 angle. By forcing the tyrosine into a g^+ χ1 angle, the phenol ring was directly pointed into what had been described in the literature to be the

ligand binding pocket (TMH3-5-6) (Kane et al., 2006), and prevented both morphine and naloxone from being able to be docked in this region. The helix chosen that is used in the final bundle has a wider turn around S4.53 and S4.54 and is shown in Figure 5 in red. Figures 5a (EC view) and 5b (side view) show the output structures (white) superimposed onto TMH4 of the β 2-AR template (lime), the TMH4 originally picked is shown in purple, the second possibility in blue and the helix used in the final bundle is shown in red.

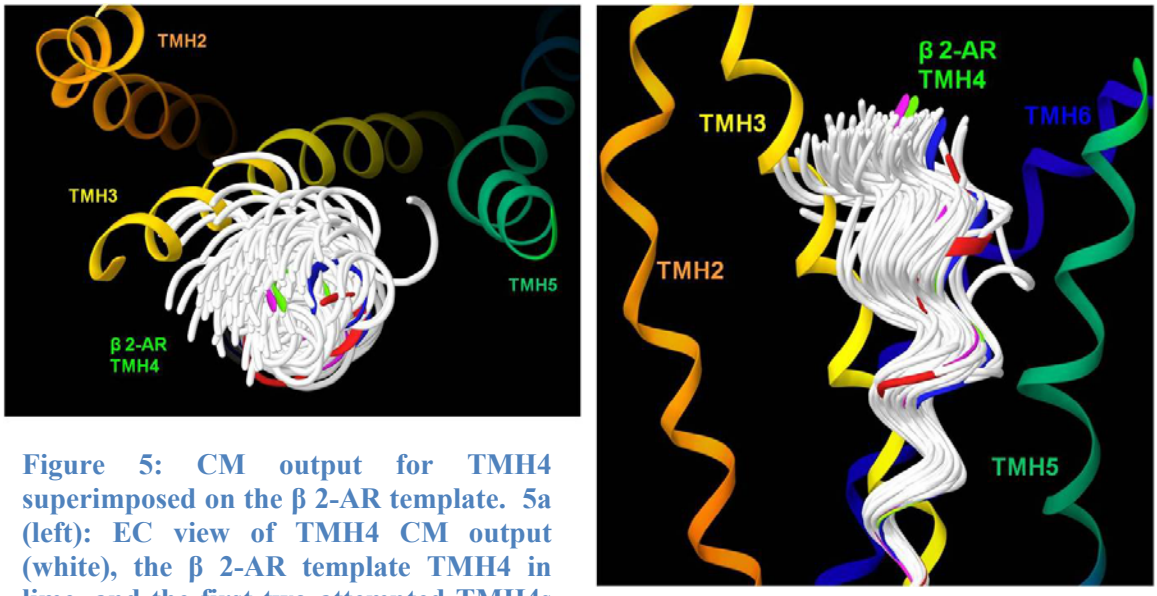


Figure 5: CM output for TMH4 superimposed on the β 2-AR template. 5a (left): EC view of TMH4 CM output (white), the β 2-AR template TMH4 in lime, and the first two attempted TMH4s in fuchsia and blue. The red helix is the final TMH4 selected. 5b (right): Side view of TMH4 CM output structures superimposed on β 2-AR template.

TMH6

Previous studies have shown that the W6.48 adopts a g^+ χ1 torsion angle in the R state and a *trans* χ1 in the R* state (Ballesteros et al., 2001; Krystek et al., 2006; Warne et al., 2008). In order to design an accurate wild type R model, the CM output structures were sorted based on the W6.48 χ1 torsion angle before superimposition onto the template structure. The output structures with the W6.48 χ1 in g^+ (12 of 105) were superimposed using the Cα atoms of R6.31 to V6.46. The intracellular end of TMH6 in the R state was chosen for superimposition since previous studies have shown that TMH6 rotates away from TMH3 during activation (Ballesteros et al., 2001) and undergoes a conformational change during activation as well (Farrens et al., 1996; Ghanouni et al., 2001; Javitch et al., 1997; Jensen et al., 2001; Lin and Sakmar, 1996; Nakanishi et al., 2006). Given that the superimposition was performed on the intracellular end of TMH6, the ionic lock area was preserved for the CM output helices. While the original helix chosen, shown in Figure 6 in fuchsia, fit into the bundle, the W6.48 residue was rotated somewhat closer to TMH5 than in the β 2-AR template structure (Figure 6, yellow W6.48). W6.48 was not included in the superimposition due to the desire not to bias the extracellular end of the bundle. However, it remained important for the location of W6.48 to be similar to that in the β 2-AR structure. As a result, a different helix was chosen from the CM output, shown in orange in Figure. 6. Figure 6 shows the CM output helices in lime superimposed onto the β 2-AR template in yellow. Asp 3.32 is also shown in Figure 6 due to its proximity to W6.48 and importance as the counter-ion. The

fuchsia helix and W6.48 is the original helix chosen for the wild type R bundle, and the orange helix and W6.48 represent the helix in the current WT R state MOR model.

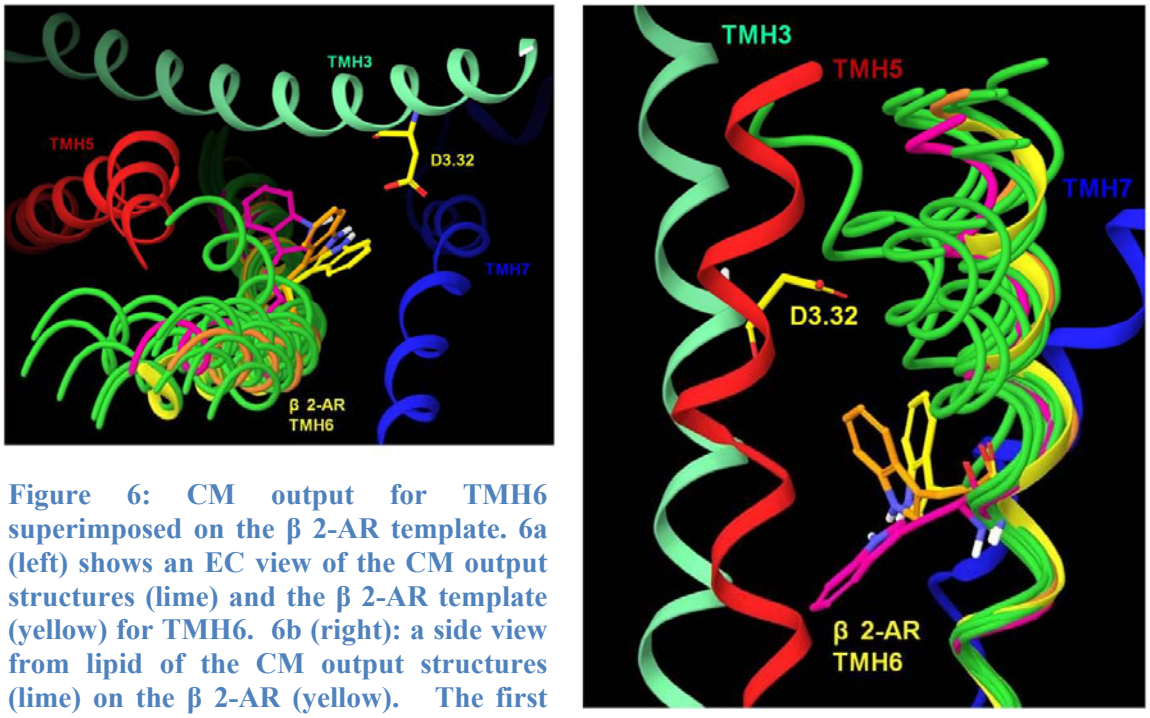


Figure 6: CM output for TMH6 superimposed on the β 2-AR template. 6a (left) shows an EC view of the CM output structures (lime) and the β 2-AR template (yellow) for TMH6. 6b (right): a side view from lipid of the CM output structures (lime) on the β 2-AR (yellow). The first and final helix chosen for use are shown here in fuchsia and orange respectively.

TMH 7

Conformational Memories was used to determine a wild-type TMH7 because this helix will be of considerable importance to future MOR modeling studies as this is the site for two of the three mutations (T7.44A and C7.47S) that changed the MOR phenotype for naloxone (Claude-Geppert et al., 2005). The output helices were superimposed on the β 2-AR template from T7.44 to L7.56, on the intracellular end. By superimposing on this area, both the conserved NPXXY regions, as well as the sites of

the two mutated residues to be examined in the future are included. Figures 7a (EC view) and 7b (side view from lipid), below, show the CM output structures in white, the β 2-AR template TMH 7 in yellow and the TMH 7 chosen for the wild type bundle in cyan.

Figure 7: CM output for TMH7 superimposed on the β 2-AR template. 7a (left) EC view of the CM TMH7 (white) with the β 2-AR template (yellow) and the selected TMH7 in cyan; 7b (right) a side view from lipid.

Once this helix had been determined, the complete bundle was put together and minimized. However, upon comparison with the β 2-AR crystal structure and the previous wild-type R bundle based on rhodopsin, it was apparent that the tyrosine at 7.43 which normally points into the bundle did not do so in the MOR model. Figure 8 shows an EC view (8a) and a side view from lipid (8b) of Y7.43 in the β 2-AR crystal structure (yellow) and the TMH 7 CM output structure (cyan). As shown, Y7.43 points outward between helices 1 and 7 instead of into the bundle as seen in the crystal structure.

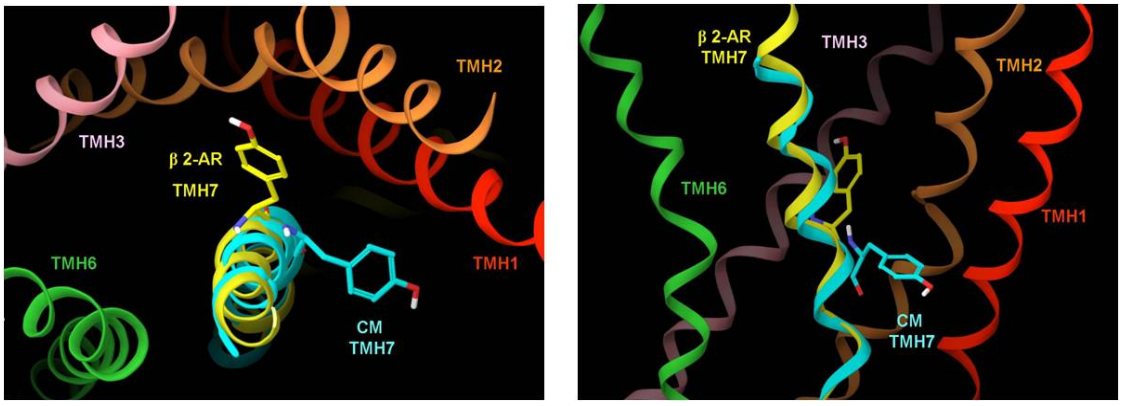


Figure 8: View of Y7.43 from the CM TMH7 superimposed onto the β 2-AR crystal structure. 8a (left) shows an EC view of Y7.43 in the β 2-AR crystal structure (lime) and the CM TMH 7 (cyan) from the WT MOR bundle. 8b (right) shows a side view from lipid.

The position of residues at 7.43 in the rhodopsin (a lysine) and adenosine A2A (a histidine) crystal structures were compared to aid in the determination of the proper direction of Y7.43 in the MOR sequence. In both of these crystal structures, as well as the β 2-AR crystal structure, the residue located at 7.43 points into the bundle. Re-examination of the CM output structures revealed that none of the CM output structures had Y7.43 pointing inwards. Since previous work had demonstrated that both morphine and naloxone lost affinity for the MOR when Y7.43 was mutated to a phenylalanine (Mansour et al., 1997), it is likely that Y7.43 faces into the bundle. Also complicating the TMH7/elbow/Hx8 region was that the elbow region of the β 2-AR template structure only had one residue, whereas the MOR sequence has two residues. Due to this, other crystal structures were examined and the decision was made to use the adenosine A2A crystal structure (Jaakola et al., 2008) for the TMH 7/elbow/Hx8 region of the MOR model.

The adenosine A2A receptor is a Class A GPCR with the conserved NPXXY motif in TMH7, as well as two residues in the elbow region connecting TMH 7 and Hx8 (Jaakola et al., 2008). Due to the structural similarities, it was determined that it would be acceptable to mutate the A2A sequence to the MOR sequence for this region. Therefore the adenosine A2A TMH7 was superimposed onto the β 2-AR template TMH7, and then mutated to the MOR sequence. The problem with mimicking the location of the 7.43 residue in the known crystal structures versus the Conformational Memories output structures appears to be that TMH7 is not completely alpha-helical. The crystal structures of the β 2-AR and A2A receptor have water in the middle of their TMH bundles, as well as an area of 3.10 helix. The CM protocol used here assumed, however, that TMH7 was solely an alpha-helix.

Preparing the WT MOR Bundle for Minimization

Once all the helices had been selected from the CM output, and the other helices had been mutated to the MOR sequence, the bundle was pulled apart 3Å from TMH 3 which is the central helix in the TMH bundle due to its extreme tilt. Since D3.32 was identified to be the counter-ion for the two charged ligands being docked, it was appropriate to pull the bundle apart away from TMH3 and attempt to resolve all steric problems with regards to that specific helix. Initially two different bundles were made based on the conformations of a series of aromatic residues on TMH3; Y3.33, F3.37 and F3.41. Both of these bundles were minimized, which resulted in two templates thought

to be suitable for docking. However, docking attempts proved unsuccessful, and some residues appeared to have questionable conformations.

Using sequence comparisons between the adenosine, rhodopsin and the β 2-AR crystal structures, it became clear that some of the residues that had initially presented conformational problems could be resolved by comparison with these three GPCR structures. Table 1 shows specific residues that were compared, the sequence to which they were compared and the orientation of the specific residue based on the models available.

Table 1: Residue comparisons between the MOR sequence and other GPCRs. This includes the Rhodopsin (Rho), the β2AR, and the Adenosine A2A (A2A) structures.

MOR Residue	Comparable Residue & Sequence(s)	Orientation of Comparable Residue	Notes
N 1.50	N—β2AR	g^+	
Y 2.42	F—A2A/ β2AR	g^+	
Y 3.41	F—A2A	g^+	
W 4.50	W—A2A/ β2AR	g^+	
F 5.43	F—Rho	g^+	
F 5.47	F—Rho/ β2AR	*Trans*	
Y 5.58	Y—A2A/ β2AR	A2A→g^+ β 2-AR →*trans*	Both Pointed Into Bundle Between TMH3/TMH6
W 7.35	W— β2AR	*Trans*	
N 7.45	N—A2A/ β2AR	*Trans*	
N 7.49	N—A2A/ β2AR	g^+	

Once the changes to the residues in Table 1 were made to the pulled apart bundle, and the toggle switch and the ionic lock residues were in the correct orientation, the new bundle was minimized in three steps as described previously. Figure 9 shows a picture of the minimized bundle including the important motifs. The toggle switch residues, C6.47, W6.48 and H6.52 are in purple, the ionic lock residues, R3.50 and T6.34 are in cyan, Y7.43 is in lavender, D3.32 (the counter-ion) is in lime, and the aromatics on TMH 3 (Y3.33, F3.37 and F3.41) are in orange.

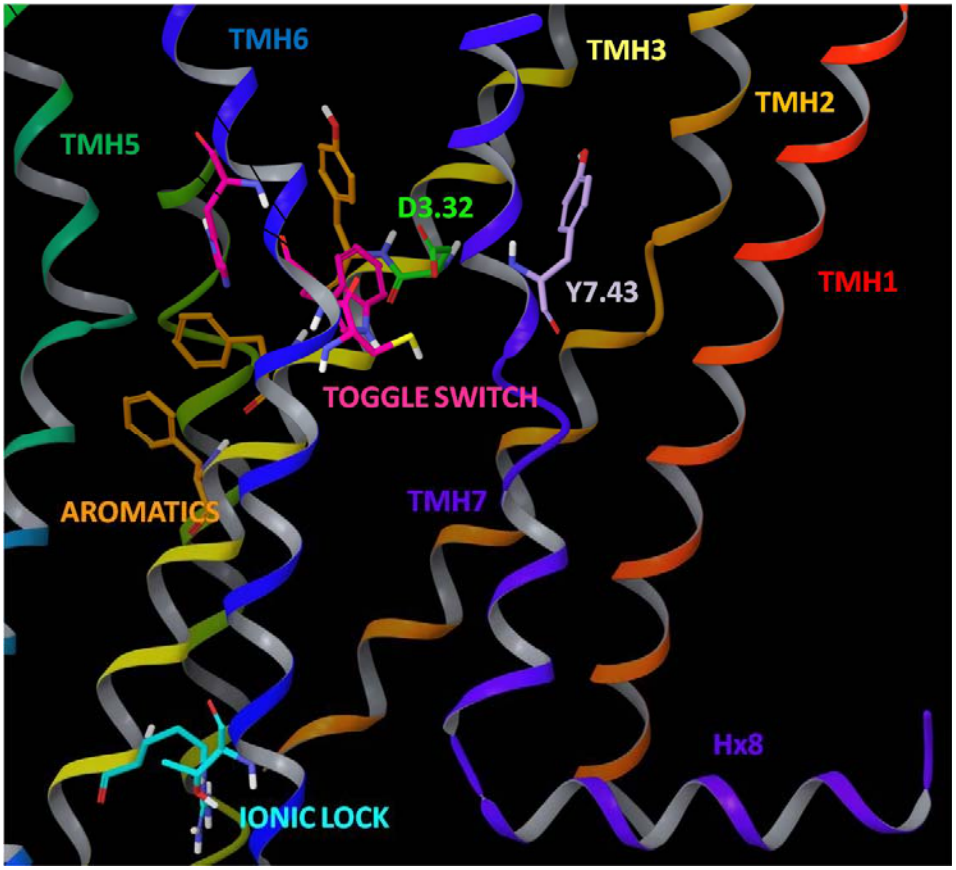


Figure 9: A minimized WT MOR R state bundle. Shown above is a view from the lipid bilayer of the minimized MOR wild type R state bundle. The ionic lock between R3.50 and T6.34 is shown in cyan, toggle switch residues on TMH 6 are shown in purple, the counter-ion for the binding of both morphine and naloxone D3.32 is shown in lime, the hydrogen bonding residue Y7.43 is in lavender and the aromatics on TMH3 are in orange.

After the creation of the MOR R state model, the model was assessed for regions into which opioid alkaloid ligands could be docked. This assessment revealed that the TMH 3, 5, 6 and 7 region could not accommodate alkaloid ligands. This result is in contrast to a previous Rhodopsin-based modeling study (Kane et al., 2006). Instead, the TMH 2, 3, 6, and 7 region appeared open for alkaloid binding. To dock the ligands, the bundle was pulled apart 2Å in all directions. This was different from the initial model minimizations in which the bundle was pulled away from TMH3. In this instance, the goal was to enlarge the center of the receptor to aid in docking the ligands, whereas before the goal was to alleviate steric clashes between side chains on the helices. Naloxone was docked using D3.32 as the primary interaction site. Once naloxone was docked and minimized, morphine was superimposed onto naloxone using the carbon atoms and the charged nitrogen atom to ensure proper placement into the starting structure. Figure 10 shows an extracellular view of the starting placement of both naloxone (fuchsia) and morphine (cyan) in the 2Å pulled apart bundle. The minimizations proceeded with the three step process described above.

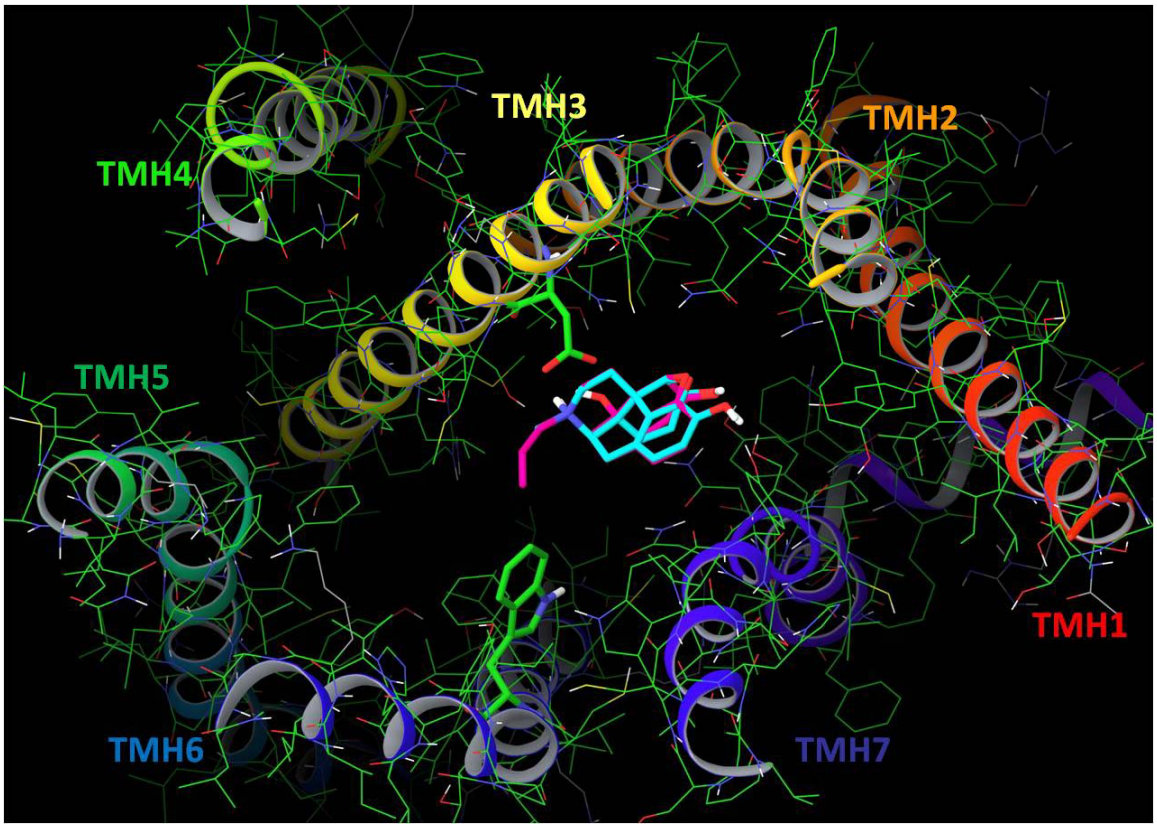


Figure 10: A WT MOR R state model with initial positions of morphine and naloxone docked. Shown above from an EC view of the MOR R state model (pulled apart 2Å) with the initial positions of both morphine (cyan) and naloxone (fuchsia) overlaid.

WT MOR Bundles with Naloxone and Morphine Docked

Once the morphine/MOR and naloxone/MOR complexes were energy minimized, they were both analyzed for key interactions. Figures 11 and 12 illustrate the final minimized ligand/receptor complexes with naloxone in fuchsia (Figure 11) and morphine in cyan (Figure 12).

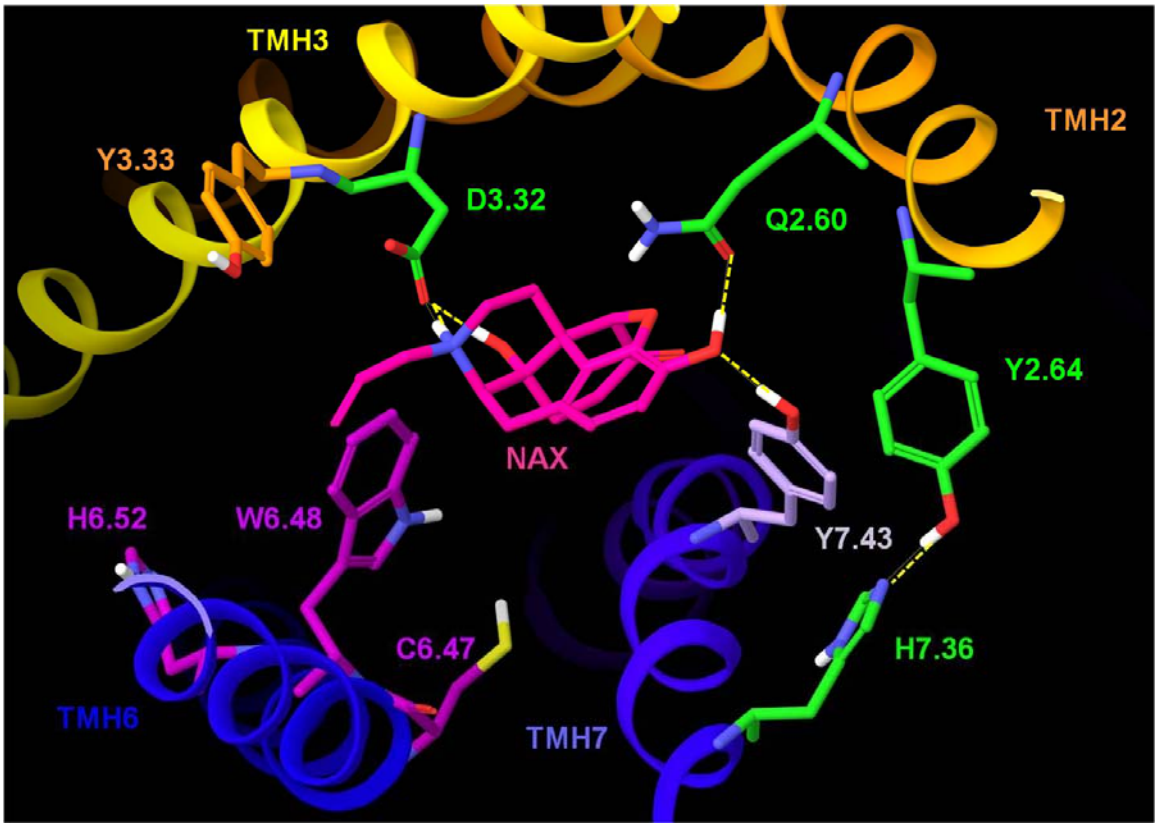


Figure 11: Naloxone (NAX; fuchsia) docked and minimized in the WT MOR R state model. Hydrogen bonds between the side chains of the MOR and naloxone are shown with yellow dashed lines.

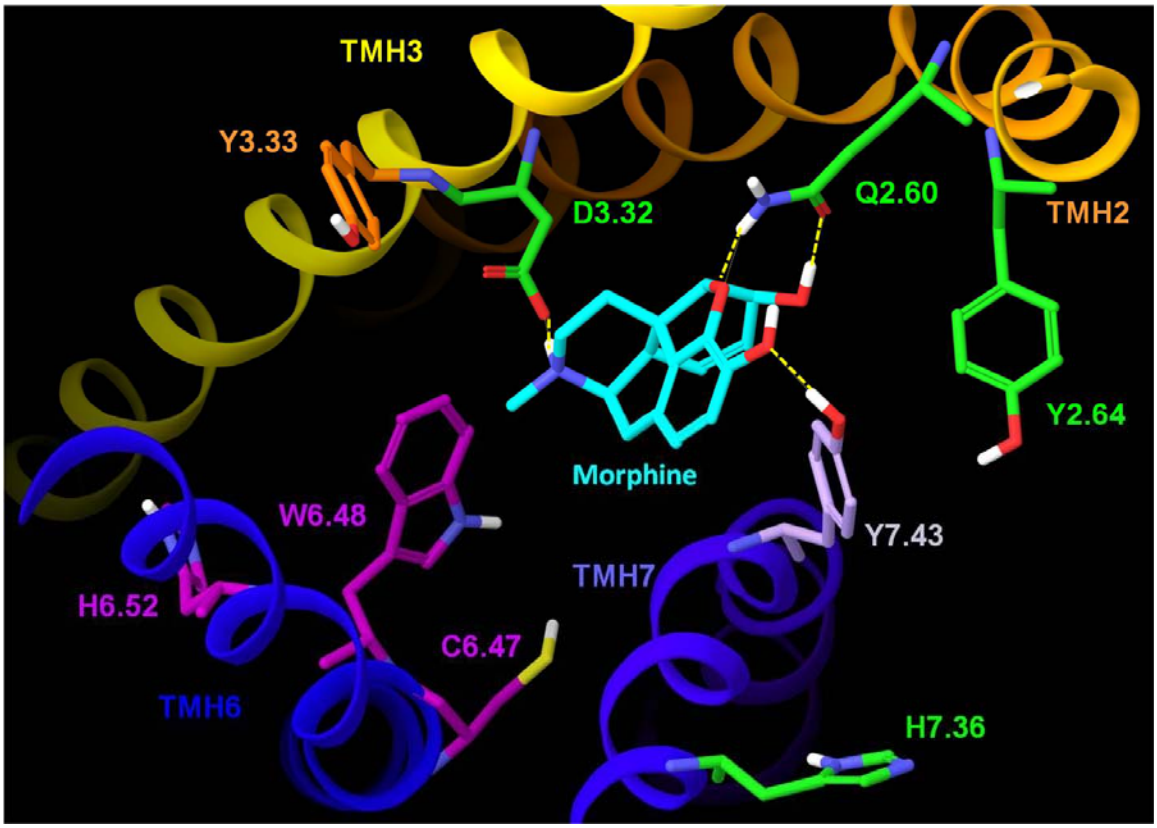


Figure 12: Morphine (cyan) docked and minimized in the WT MOR R state model. Hydrogen bonds between side chains and morphine are shown with yellow dashed lines.

The yellow dashed lines indicate hydrogen bonds formed between the MOR and each ligand. The docking studies revealed that naloxone and morphine both dock in the same region of MOR, the TMH2-3-6-7 region. Each ligand can establish hydrogen bonding interactions with the MOR and the sets of residues involved in these interactions between the naloxone/MOR and morphine/MOR are partially overlapping. Naloxone forms two hydrogen bonds with D3.32, one with the NH^+ nitrogen and another with the C ring hydroxyl group. Morphine however, only forms one hydrogen bond with D3.32, via the NH^+ nitrogen. The hydroxyl that forms the second hydrogen bond with D3.32 in

naloxone is a non-polar hydrogen in morphine off of the C ring (see Figure 2). On the other hand, morphine forms two hydrogen bonds with Q2.60 with both the oxygen in the tetrahydrofuran ring and the C ring hydroxyl, but naloxone only forms one hydrogen bond with Q2.60 via it's a ring hydroxyl. Both morphine and naloxone have a hydrogen bond between Y7.43 and the A ring hydroxyl group in the docking studies reported here. In the naloxone dock, neither the tetrahydrofuran (THF) oxygen nor the carbonyl oxygen on the C ring has a hydrogen bond. Tables 2 and 3 provide information about the geometries of the hydrogen bonds formed in these two complexes. The superscript "a" and "d" designate the residue or atom as the acceptor (a) or donor (d) in each hydrogen bond. It is clear that the geometries of these hydrogen bonds are nearly linear and that the hydrogen bonding distances are quite good.

Also shown in the naloxone dock in Figure 11, Y2.64 and H7.36 form a hydrogen bond with each other in the naloxone/MOR complex, as indicated with the yellow dashed lines (173.2°, 2.689Å). However, in the morphine dock (Figure 12), this does not occur. The distance from the Cα atoms of Y2.64 and H7.36 is 12.3Å in the naloxone dock, and 16.3Å in the morphine dock. As shown in Figure 12, morphine has rotated itself such that it forms hydrogen bonds with atoms that naloxone does not. As a result, it seems based on visual inspection that the EC end of TMH7 is pushed out of the bundle to some degree in the morphine/MOR complex, as evidenced by the loss of a hydrogen bond between Y2.64 and H7.36.

Table 2 Hydrogen Bond Atoms, Angles and Distances for the Naloxone/MOR Complex

Residue	Atom of Naloxone	Angle of H-Bond	Heteroatom-Heteroatom Distance
Y7.43 [d]	O [a]—A Ring	174.4°	2.566Å
Q2.60 [a]	OH [d]—A Ring	170.9°	2.565Å
D3.32 [a]	NH^{+} [d]	171.2°	2.529Å
D3.32 [a]	OH [d]—C Ring	178.0°	2.383Å

Table 3 Hydrogen Bond Atoms, Angles and Distances for the Morphine/MOR Complex

Residue	Atom of Morphine	Angle of H-Bond	Heteroatom-Heteroatom Distance
Y7.43 [d]	O [a]—A Ring	175.3°	2.634Å
Q2.60 [d]	O—THF	158.9°	2.687Å
Q2.60 [a]	OH [d]—C Ring	177.4°	2.575Å
D3.32 [a]	NH^{+} [d]	174.3°	2.505Å

The central hypothesis of this project is that the N-allyl substituent of naloxone sterically blocks the movement of the χ1 angle of W6.48 from g^{+}→*trans*, thus preventing the WT MOR R state receptor from becoming activated. Figure 11 illustrates that in the naloxone/MOR complex, the naloxone N-allyl substituent is positioned to prevent the W6.48 χ1 from a g^{+}→*trans* movement. Figure 13 depicts how the naloxone N-allyl group is prevented from adopting an alternate conformation by Y3.33. Shown in this figure are naloxone (fuchsia), W6.48 (purple) and Y3.33 (orange) in Van der Waals representations. The yellow stick tryptophan residue illustrates the position of W6.48 if

its χ1 undergoes the g^{+}→*trans* movement. This figure illustrates that the location of Y3.33 prohibits any movement of the naloxone N-allyl, thus restraining the movement of W6.48 and keeping the MOR in an inactive, R state.

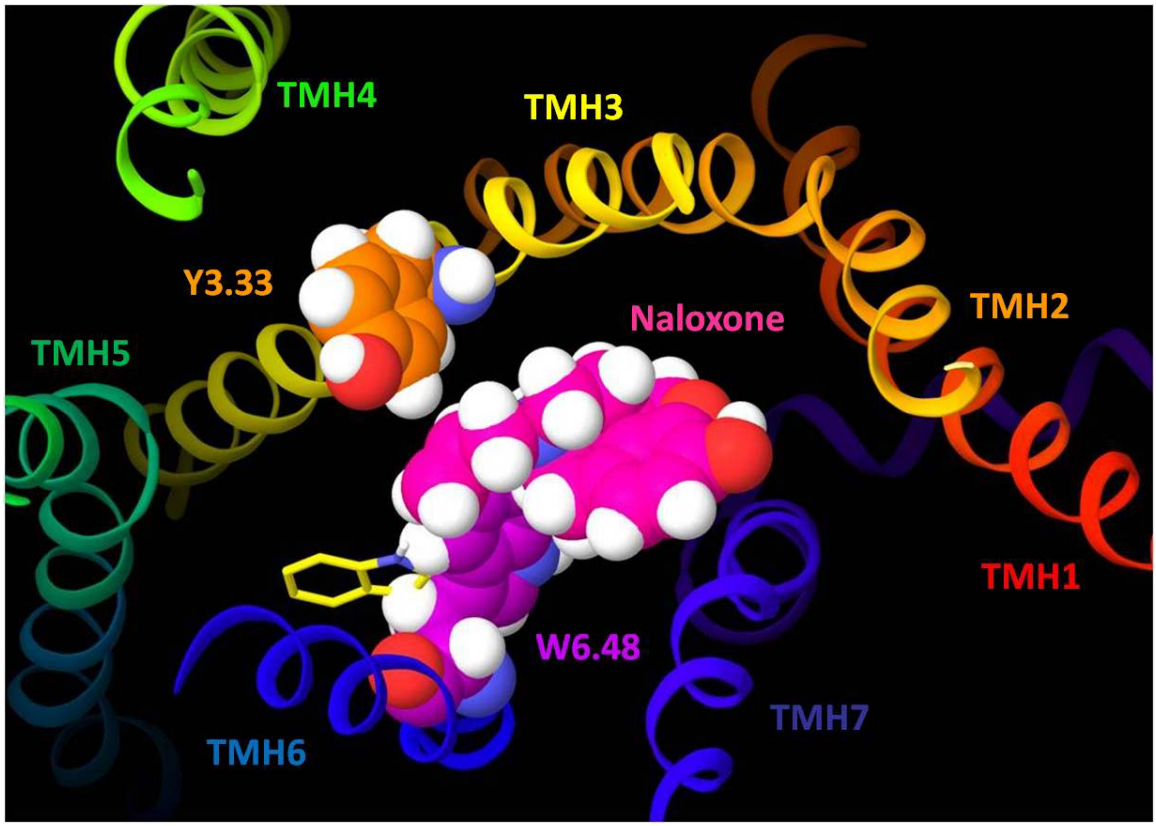


Figure 13: Naloxone (fuchsia), W6.48 (purple) and Y3.33 (orange) in VDW representations in the WT MOR model. The yellow tube representation depicts the position that W6.48 would assume if its χ1 angle changed to *trans*. This figure illustrates that the naloxone N-allyl substituent prevents the W6.48 χ1 g^{+}→*trans* movement and that Y3.33 restricts the naloxone N-allyl substituent to its current position.

The final docked positions of naloxone and morphine are supported by the mutation literature which suggests that D3.32 (Surratt et al., 1994) and Y7.43 (Mansour et al., 1997) are important for both naloxone and morphine binding. For the hypothesis tested here to be supported, morphine should **not block** W6.48 from changing its χ1 angle in the energy minimized morphine/MOR complex. The N substituent in morphine (methyl group) is much smaller in size compared to that of naloxone (allyl group), see Figure 2. Figure 12 illustrates morphine in an energy minimized MOR bundle. Here it is clear that the N substituent would not block the W6.48 χ1 $g^{+}\rightarrow$*trans* movement.

Interaction Energies

The interaction energies between each docked ligand and the MOR were calculated using the final minimized docks. The electrostatic, Van der Waals (VDW) and total energy of all residues within 5Å of the ligands were calculated and are shown in Tables 4 (naloxone) and 5 (morphine). As shown for both ligands, the residue with the highest single contribution to the interaction energy is D3.32, the counter-ion. Hydrogen bonds with Q2.60 and Y7.43 also make significant contributions to the interaction energies for both ligands.

Table 4: Interaction Energies between Naloxone and Residues within 5Å of a MOR Binding Site

	Electrostatic kJ/mol	VDW kJ/mol	Total Energy kJ/mol
W 6.48	3.1645	-10.5648	-7.4003
N 7.45	-0.2580	-0.8216	-1.0796
V 3.28	0.2226	-5.8006	-5.5780
Y 6.54	1.3465	-1.0881	0.2584
L 2.57	-0.4249	-7.5177	-7.9426
I 6.45	1.7966	-7.8247	-6.0281
Y 1.39	-2.6868	-1.1139	-3.8007
D 3.32	-364.4160	62.6916	-301.7244
I 7.39	-0.6574	-8.3623	-9.0197
G 7.42	2.4788	-5.9857	-3.5069
Y 2.64	0.5300	-0.3670	0.1630
W 7.35	-0.1730	-0.4675	-0.6405
Q 2.60	-36.6522	3.6969	-32.9553
Y 3.33	-0.2129	-5.4663	-5.6792
V 6.55	1.7830	-2.5514	-0.7684
M 3.36	-1.2174	-3.0189	-4.2363
I 3.29	0.7937	-8.4107	-7.6170
Y 7.43	-59.6656	2.6797	-56.9859
S 7.46	-1.4809	-0.7276	-2.2085
			Total Energy: -456.75kJ/mol

Table 5 Interaction Energies between Morphine and Residues within 5Å of a MOR Binding Site

Residue	Electrostatic kJ/mol	VDW kJ/mol	Total Energy kJ/mol
W 6.48	0.2537	-5.7103	-5.4566
N 7.45	1.0007	-0.5258	0.4749
C 3.25	-6.4965	-1.1660	-7.6625
I 3.29	-1.2516	-8.1245	-9.3761
I 6.45	3.3759	-5.6163	-2.2404
Y 1.39	-3.7717	-2.4016	-6.1733
Q 2.60	-58.8832	9.6861	-49.1971
I 7.39	-1.9547	-9.4002	-11.3549
D 3.32	-235.2808	22.5292	-212.7516
G 7.42	-0.8948	-7.5533	-8.4481
V 3.28	-0.0483	-6.9045	-6.9528
Y 3.33	-1.0251	-1.8050	-2.8301
C 7.38	-4.9418	-0.7526	-5.6944
V 1.42	0.0017	-0.2514	-0.2497
L 2.57	0.4266	-4.1771	-3.7505
Y 7.43	-49.3070	-9.2397	-58.5467
S 7.46	-0.8364	-0.5910	-1.4274
			Total Energy: -391.64 kJ/mol

Summary

A wild type MOR R state model was constructed using the β 2-AR and adenosine A2A crystal structures as templates along with modeled changes to TMH2, TMH4 and TMH6 based on output from Conformational Memories calculations. The ionic lock between R3.50 and T6.34 in the MOR model is formed in the unoccupied MOR model and with naloxone present. The binding pocket toggle switch determined to be present in the β 2-AR structure, is likely also present in the MOR model, as the same residues are present at 6.47 (cysteine) and 6.48 (tryptophan), and both sequences have aromatic residues at 6.52 (phenylalanine in β 2-AR and histidine in MOR). The central hypothesis of this project is supported by the final morphine/MOR and naloxone/MOR complexes. In these complexes the N-allyl group of naloxone blocks the movement of the χ1 angle of W6.48, but the corresponding substituent in morphine, an N-methyl, does not block the movement of W6.48. Key residues involved in the binding of both ligands were found to be the counter-ion D3.32, as well as hydrogen bonding residues Q2.60 and Y7.43. The work presented here forms the basis for future studies of the MOR.

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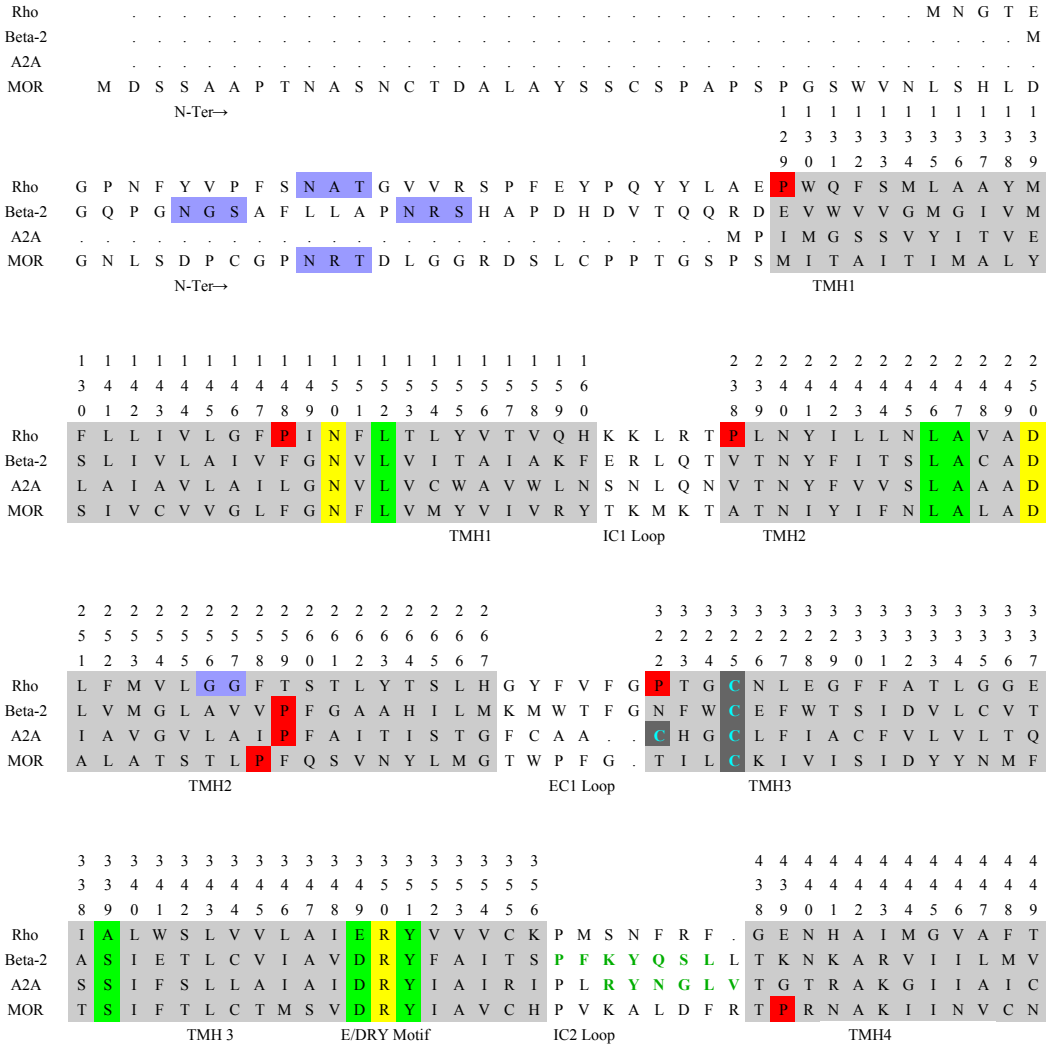
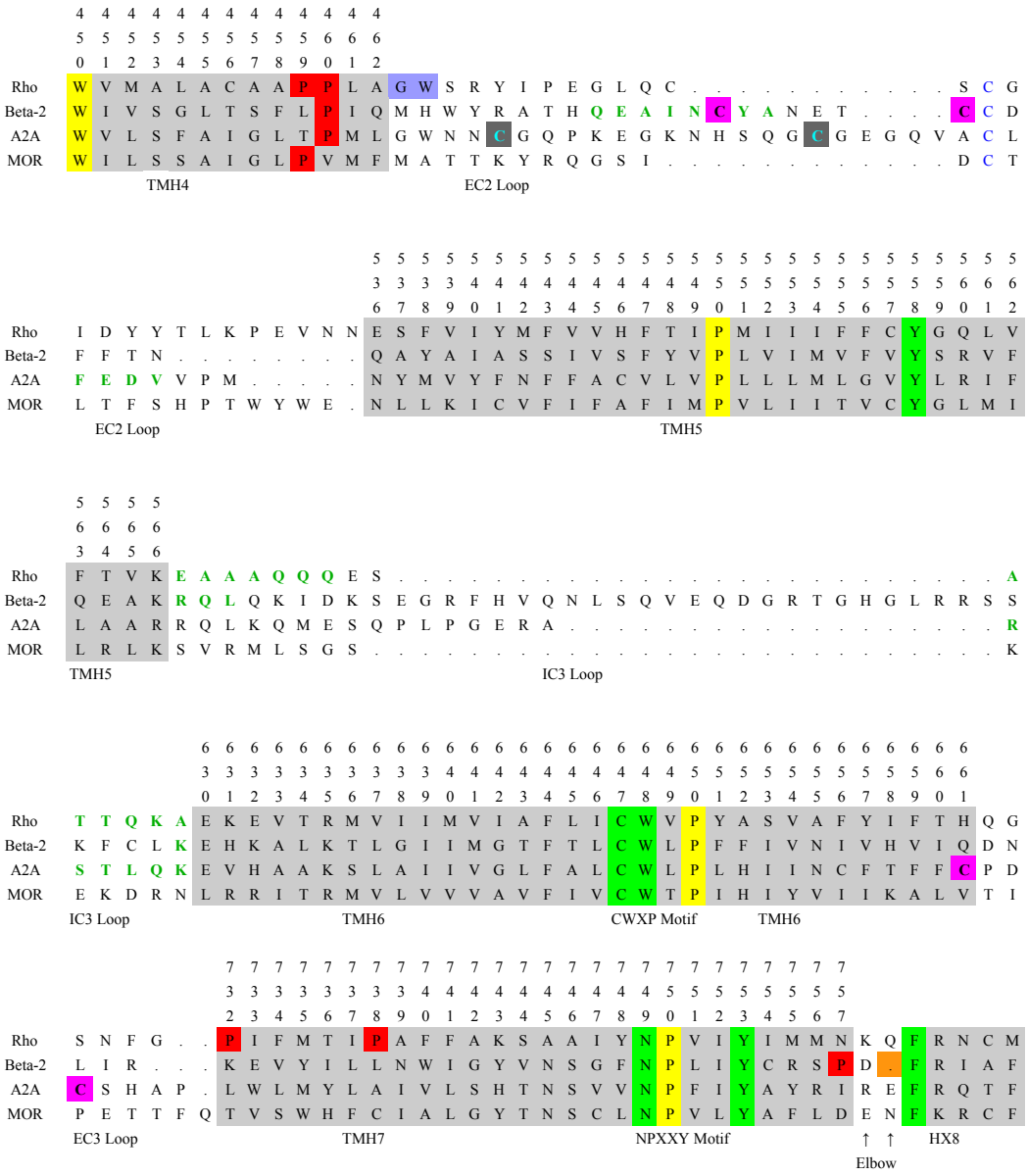
APPENDIX A

Sequence Alignment



APPENDIX A

Sequence Alignment



APPENDIX A

Sequence Alignment

Rho L T T I C C G K N P L G D D E A S A T V S K T E T S Q V A P A . . . .
Beta-2 Q E L L C L R R S S L K A Y G N G Y S S N G N T G E Q S G Y H V E Q E K E N K
A2A R K I I R S H V L R Q Q E P F K A A G T S A R V L A A H G S D G E Q V S L R L
MOR R E F C I P T S S N I E Q Q N S T R I R Q N T R D H P S T A N T V D R T N H Q
C-Ter→

Rho . . . .
Beta-2 L L C E D L P G T E D F V G H Q G T V P S D N I D S Q G R N C S T N D S L L
A2A N G H P P G V W A N G S A P H P E R R P N G . . . .
MOR L E N L E A E T A P L P . . . .
C-Ter→

Key

Sequence	Uniprot ID
Rho	P08100
Beta-2	P07550
MOR	P35372
A2A	P29274

Colors in TMH portions

(red)	Prolines
(yellow)	Highly conserved
(green)	Reasonably conserved
(orange)	Loop gap warning (only used in TMH7 to HX8 elbow)
(lavender)	Assorted Motifs: N-ter glycosylation sites, GG motif, and GW motif
C	In a disulfide bridge
C	C3.25 disulfide bridge from EC2 Loop
C	Internal loop disulfide bridge
X	Loop w/helical secondary structure
S	Possible phosphorylation site
C	Palmitoylation site

Glycosylation Motif is NXS or NXT, and X can't be a Proline